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Gamma Ray Inactivation Studies on Some Functional Elements of HVJ

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SUMMARY

HVJ (hemagglutinating Virus of Japan) isolated from mice shows particular characteristics in the MNI group viruses. For the better understanding of the characteristics of this virus the size of the sensitive parts bearing various activities were determined by gamma ray irradiation.

It was shown by gamma ray irradiation that the sensitive volumes of infective, and hemagglutinating activities were 38 millimicrons, and 10 millimicrons, respectively in diameter as spheres. It is of interest that the rate of inactivation of hemagglutinating activity coincides with that of loss of hemolytic activity. The sensitive volume of the infectivity of this virus is comparable with that of influenza virus, PR8 strain.

INTRODUCTION

Several years ago HVJ was isolated from mice in our laboratory and was temporarily called HVM, Z strain. Some of its biophysical properties including its morphology were described in a previous paper (Fukai and Suzuki, *1955b*). The hemolytic activity which is also characteristic to some viruses of the MNI group was also described in the same report. Hosaka analized the mechanism of the hemolysis of this virus and pointed out that there is a decrease in sphingomyelin in red blood cells in the process as with the mumps virus. Okada reported on the fusion phenomenon of Ehrlich's ascites tumor cells using concentrated purified HVJ *in vitro* and cells shaken at 37°C. He found an intimate relationship between hemolytic activity and the cell fusing activity of this virus.

To study the above mentioned characteristics we have determined the sensitive volume of the hemagglutinating, hemolytic, and infective elements of this virus. Gamma ray irradiation and Lea's target theory were applied. This paper describes the results of these experiments.

MATERIALS AND METHODS

1. *Virus*

HVJ, Z strain, was employed throughout the work. Freshly harvested chorioallantoic fluid infected with the original strain was used as the starting material.

The virus was prepared by inoculation into the chorioallantoic cavity of 11 day old eggs incubated at 38.5°C. one fifth ml of infected chorioallantoic fluid by the routine method. The infected fluid was harvested 48 hours after incubation at 35.5°C. The virus was purified

and concentrated by two cycles of differential centrifugation using a Spinco L ultracentrifuge and was finally suspended in phosphate buffered saline.

2. *Infectivity titration*

For the infectivity titration, 10 fold serial dilutions of samples were prepared in broth containing 1,000 μ/ml of penicillin.

One fifth ml of each dilution was inoculated into the allantoic cavity of five 11 day old eggs. After 72 hours incubation at 35.5°C, eggs were chilled at 4°C to avoid bleeding and the chorioallantoic fluid of individual eggs was tested for virus growth by hemagglutination on slides. The 50 per cent infective titer (EID_{50}) was calculated by the method of Reed and Muench and expressed as the titer per ml.

3. *Hemagglutination titration*

To two series of serial 2 fold dilutions of the samples from 2-and 3-fold dilution, 0.5 ml of a 0.5 per suspension of thrice washed chicken red blood cells were added. The mixtures were kept in a cold room at 4°C for one and a half hours and the hemagglutinating end point was read by the sedimentation of red blood cells at the bottom of the tubes. The titers were expressed for ml.

4. *Hemolytic titration*

One ml of ice-cold virus sample was mixed with 2 ml of 2 per cent red blood cells suspension. The tube containing the mixture was kept cold for 30 minutes to allow virus adsorption onto the red cells and then kept in a 37°C water bath for 60 minutes with mechanical agitation. At the end of incubation the tube was centrifuged at 1,500 rpm for 5 minutes. The supernatant fluid was transferred to a colorimeter cuvette and the amount of hemoglobin liberated was determined in a Coleman spectrophotometer at 5,400 A. All determinations were expressed as optical densities. Readings given are those in which the control readings have been subtracted from the direct readings.

5. *Irradiation*

In a Co^{60} irradiation apparatus, a specially designed remote control attachment was used for precise adjustment of the irradiation distance. Measurements showed that the intensity of the gamma rays from the Co^{60} source was 2167 röntgen per minutes at a distance of 50 mm from the center of the source. The gamma ray dosis for the inactivation was calculated from this value.

6. *Fractionation of the virus in a sucrose density gradient column*

A modification of Schwerdt-Schaffer's method for the fractionation of poliovirus was used. The details have been described in the previous report (Yoshishita, 1959).

RESULTS

1. *Relationship between hemagglutinating activity and hemolytic activity of the virus preparation used*

The virus preparation used for irradiation experiments was preliminary tested for its hemagglutinating activity and hemolytic activity simultaneously before use.

A purified virus suspension having Ca. 4,000 HAU/ml was diluted in a two fold dilution series. Aliquots at each dilution were tested for their hemagglutinating and hemolytic activities and the results are tabulated in Table 1a. When aliquots of the diluted samples from the other series were tested after freezing and thawing them five times the values for hemagglutinating and hemolytic titration were as shown in Table 1b. Fig. 1 shows the relationship between hemagglutinating and hemolytic activity with both native and freeze-thawed virus, the relationship was linear when the two activities were plotted in log-log scale.

Fig. 1. Correlation between hemolytic activity and virus concentration (HA).

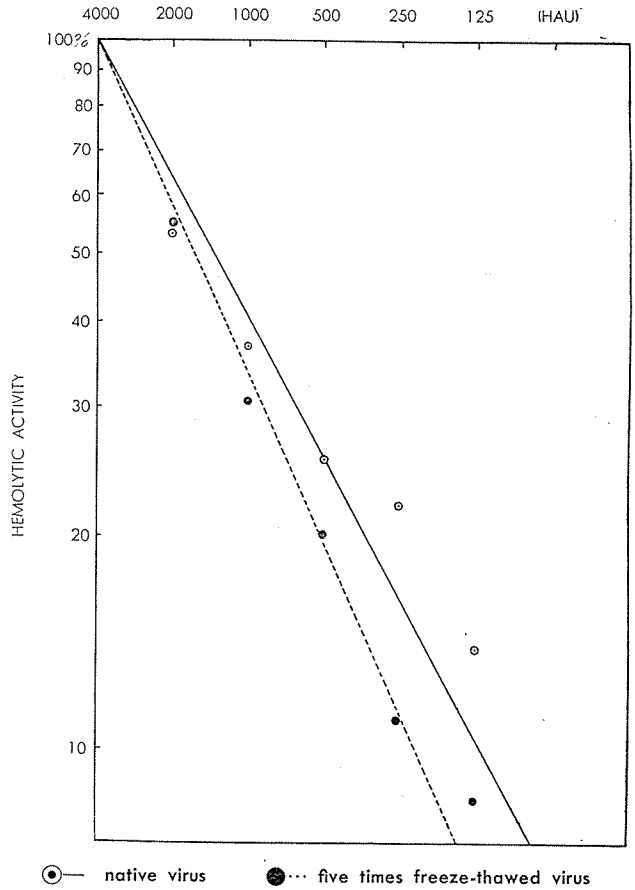


Table 1a. Relationships between hemagglutinating and hemolytic activity of native HVJ

Dilution	Hemagglutinating activity	Hemolytic activity	
		Hb liberated (O.D.)	(per cent)
2^0	4000	0.370	(100)
2^1	2000	0.190	(51)
2^2	1000	0.140	(38)
2^3	500	0.105	(28.5)
2^4	250	0.080	(22)
2^5	125	0.053	(14)

2. *Experiments on gamma ray inactivation*

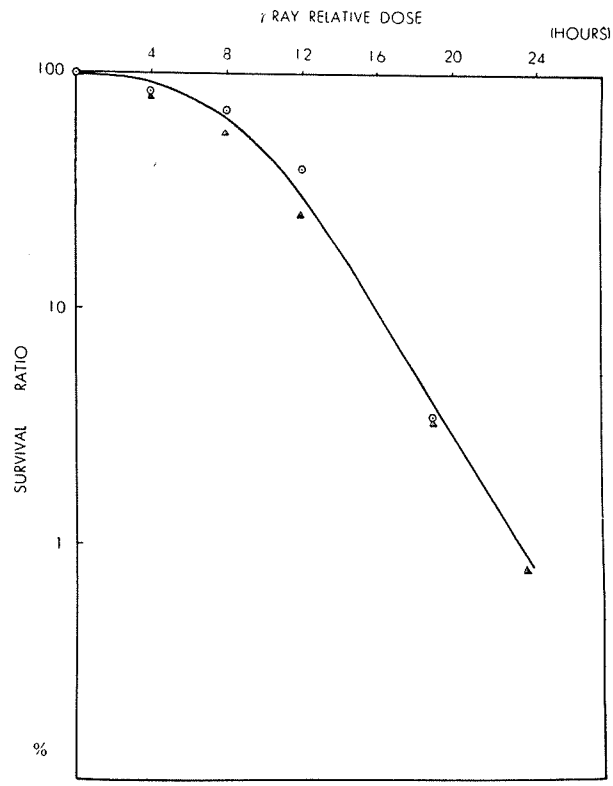
In inactivation experiments, aliquots of the samples to be irradiated were

distributed in glass ampoules and sealed. These ampoules were placed in the irradiating apparatus at appropriate positions for the purposes of the experiments. Their positions were determined by measuring the distance between the center of the gamma ray source and the middle point of the sample liquid layer.

Table 1b. Relationships between hemagglutinating and hemolytic activity ot freeze-thawed HVJ

Dilution	Hemagglutinating activity	Hemolytic activity	
		Hb liberated (O.D.)	(per cent)
2^0	4000	1.250	(100)
2^1	2000	0.650	(52)
2^2	1000	0.375	(30)
2^3	500	0.250	(20)
2^4	250	0.180	(10.4)
2^5	125	0.105	(8.5)

Fig. 2. Inactivation curves of hemagglutinating activity of HVJ suspended in broth medium.



1) *Inactivation of hemagglutinating activity*

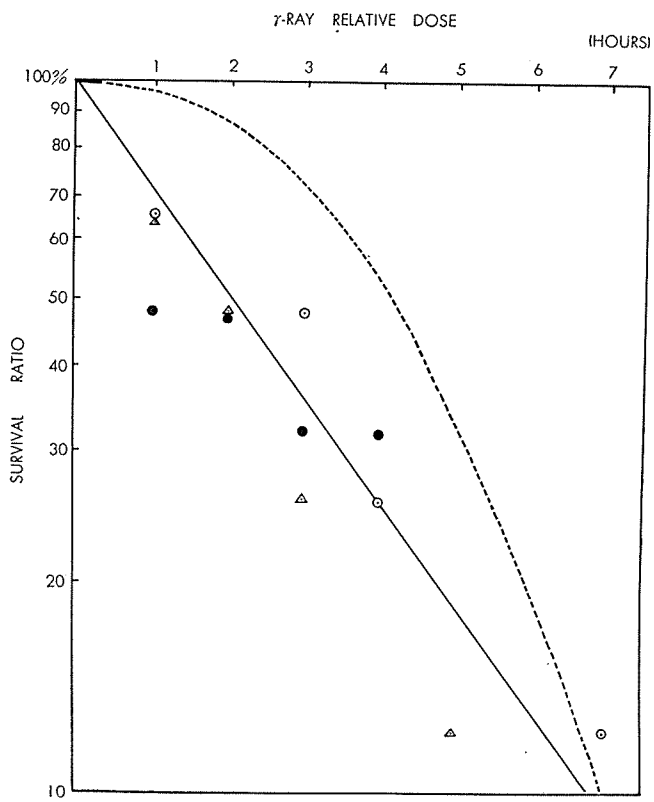
A. *Inactivation of hemagglutinating activity of virus particles*

The experiments were conducted in two ways. In the one experiments, virus suspended in phosphate buffered saline-broth mixture (1 : 9) was used and in the other freeze-dried virus was irradiated.

i) *Virus in saline-broth mixture*

One ml aliquots of the sample, sealed in ampoules, were irradiated for 4, 8, 12, 18 and 24 hours at a distance of 30 mm for the Co^{60} source. After irradiation each sample was tested for its hemagglutination titer using the pattern method. The survival ratio was calculated from the titer of each sample and the control titer. The result is presented in Fig. 2 and shows a multiple hit inactivation curve with a shoulder.

fig. 3. Inactivation of hemagglutinating activity of dried HVJ (solid line). Dotted line shows the inactivation curve in suspension. (cf. Fig. 2.)



ii) *Freeze-dried virus*

One fourth ml (40,000 HAU/ml) aliquots of the virus sample were distributed in a number of small flat bottomed aluminium cups and then freeze-dried. Then the cups were placed in the irradiating apparatus. After irradiation at a distance of 20 mm for the required time, the dried material was dissolved in distilled water and tested for its hemagglutinating titer using phosphate buffered saline as diluent. In this experiment a linear inactivation curve was obtained as shown in Fig. 3.

The difference in the shape of the inactivation curves of suspended and dried virus cannot be explained simply, but a so-called secondary effect of the irradiation may be prominent in the former case. The slope of the inactivation curve

Fig. 4. Inactivation of hemagglutinating activity of isolated HVJ hemagglutinin prepared by ether treatment.

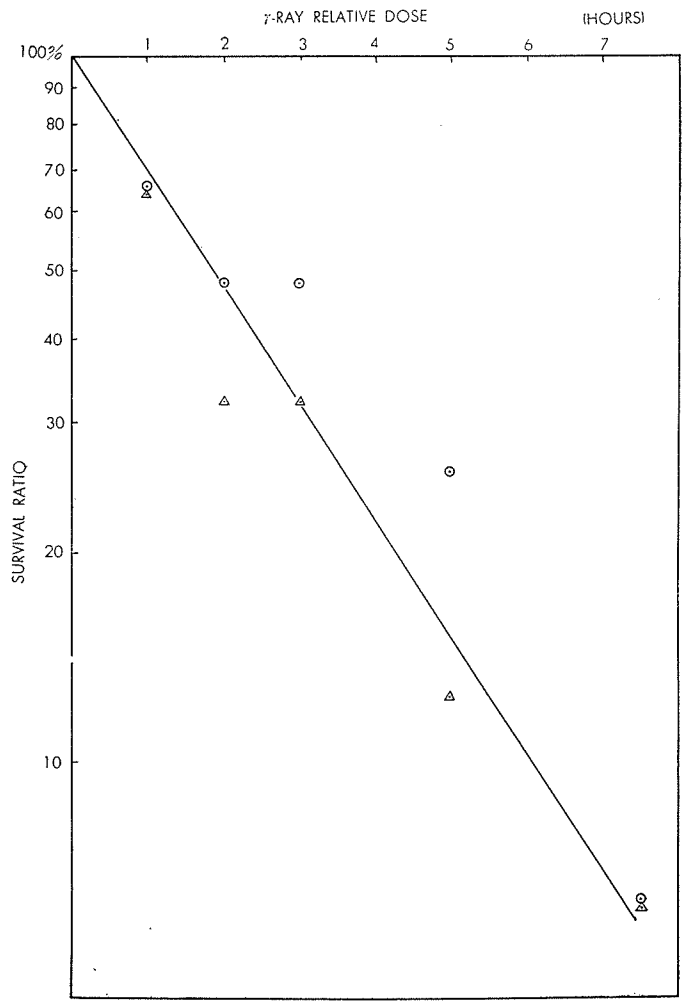


Table 2. Preparation of hemagglutinin from virus particles by Hoyle's ether treatment.

HVJ, 12800 HAU./0.5 ml × 5 ml
+ 2.5 ml anaesthetic ether
mixing by magnetic stirrer in cold ice bath
for 2 hours
low speed centrifugation

ether layer	water phase
discarded	residual ether removed by bubbling for 30 minutes
	HA : 8192 U/0.5 ml
	10 fold diluted with phosphate buffered saline
	centrifuged at 25,000 rpm for 60 minutes in Spinco L ultracentrifuge with #40 rotor
sediment	supernatant fluid
discarded	upper half of the contents of the tube were used for the irradiation experiment
	HA : 256 U/0.5 ml
	+
	equal volume of broth

of the dried virus sample was a little different from that of the linear part of the inactivation curve of the suspended virus.

B. *Inactivation of hemagglutinin prepared from virus particles by ether treament*

The purified virus suspension was treated with anaesthetic ether following Hoyle's method. The method used is summarized in Table 2. In this experiment hemagglutinin was not separated from the other complement fixing antigen.

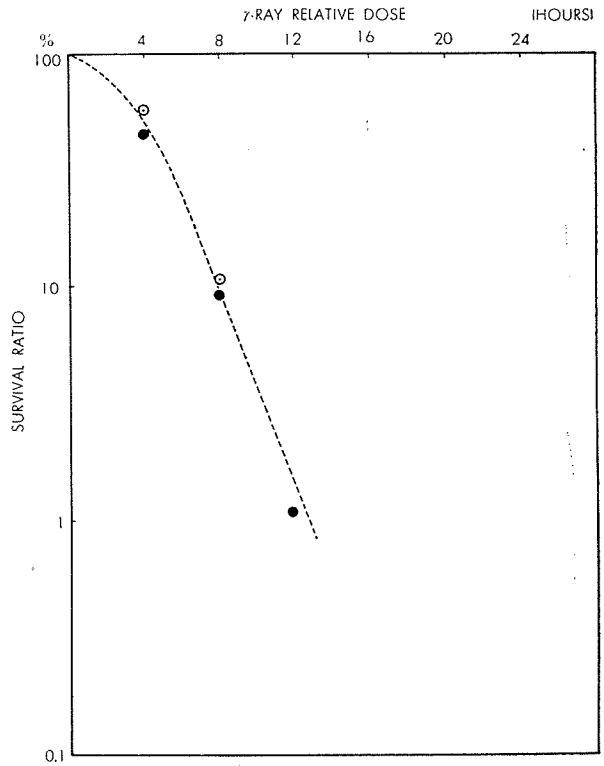
The samples were irradiated at a distance of 20 mm from the source for 1, 2, ... , 7 hours and tested for their hemagglutinating activity using the pattern method. Fig. 4 shows the result which suggest that they have the same inactivation rate as freeze-dried native virus. Regarding the curve as a single hit inactivation curve and applying the target theory, the sensitive volume of the hemagglutinating element was calculated to be 10 millimicron in diameter. In this experiment, indirect or secondary effects did not appear though hemagglutinin was irradiated in suspended state.

2) *Inactivation of hemolytic activity*

A. *Virus suspended in broth*

The virus suspension was diluted with nine volume of broth and aliquots of the diluted sample in sealed ampoules were irradiated. After irradiation for various periods, the samples were diluted ten times with phosphate buffered saline and tested for their hemolytic activity. The results are presented in Fig. 5. They suggest that the inactivation curve is not linear. As for inactivation of the hemagglutinating activity of the virus particles, the reason of the deviation from linearity must be due to the so-called secondary effect of the ionizing radiation.

Fig. 5. Inactivation curve of hemolytic activity of HVJ suspended in broth medium.



B. *Freeze-dried virus*

One fourth ml (40,000 HAU/ml) aliquots of the virus sample were frozen and cautiously dried in aluminium cups, and gamma irradiation was given. After completion of irradiation each sample was resuspended in 4 ml of distilled water. The dissolved samples were further diluted twenty-one times with phosphate buffered saline to give a suitable concentration for titration and then tested for hemolytic activity. The results are presented in Table 3. and Fig. 6. A fairly linear inactivation curve was obtained in this experiment and regarding the curve as a single hit curve the diameter of part sensitive to gamma irradiation was calculated as a sphere of 10 millimicrons diameter, assuming it had a density of 1.35. No secondary effect was observed in this case, because the sample was irradiated in the dry state.

3) *Inactivation of infectivity*

The virus sample was diluted ten fold with broth and aliquots sealed in ampoules were irradiated for 20, 40, 60, 80 and 120 minutes at 30 mm distance from the Co^{60} source. The irradiated samples were immediately titrated for surviving infectivity. The survival curve is shown in Fig. 7. The results are in good agree-

ment with the exponential equation for a single hit curve. Calculations from the 37 per cent survival doses indicate a diameter of about 38 millimicrons (as a sphere) as the size of the infective center.

Fig. 6. Inactivation of hemolytic activity of dried HVJ. Dotted line shows the inactivation curve in suspension in broth medium. (cf. Fig. 5.)

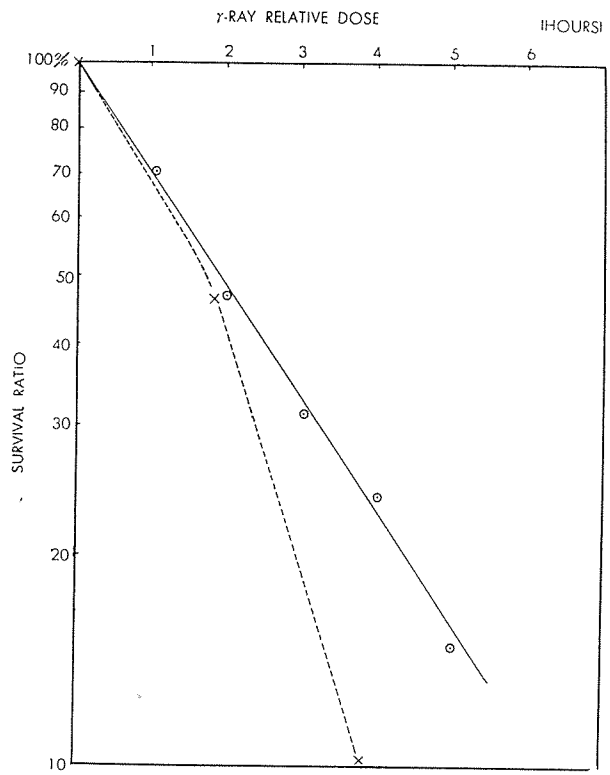
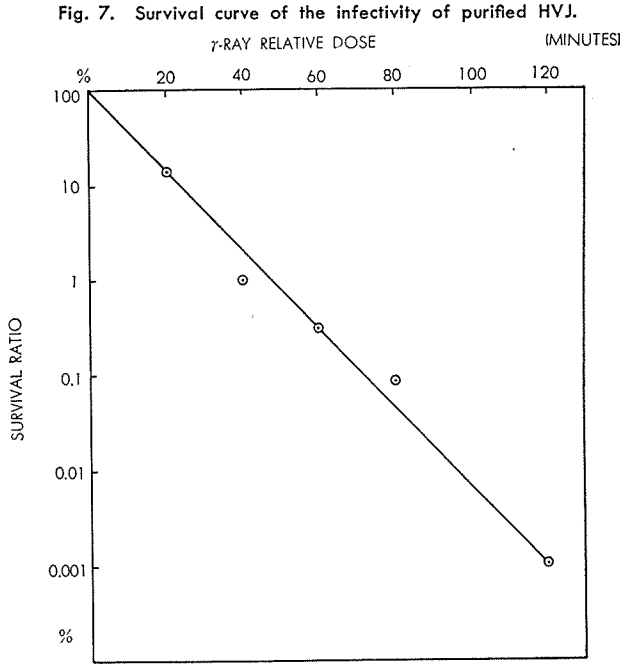


Table 3. γ-ray inactivation of hemolytic activity of dried purified HVJ

γ-ray relative doses (hours)	hemolytic activity	
	Hb liberated (O.D.)	per cent
0	0.638	100
1	0.450	70.6
2	0.300	47.0
3	0.195	30.5
4	0.153	24.0
5	0.088	13.8

Fig. 7. Survival curve of the infectivity of purified HVJ.



3. *Distribution of hemagglutinating and hemolytic activity in a sucrose density gradient column*

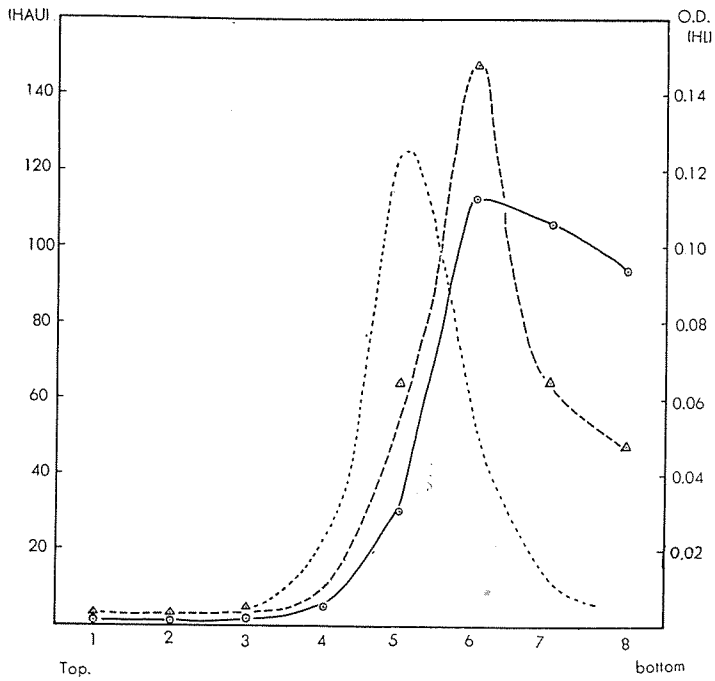
In the preceeded section the inactivation curves of some functional elements of HVJ were presented. To check the homogenity of the virus preparation used, the localization of the virus entities in a sucrose density gradient column after centrifugation was studied. The purified virus suspension was layered on the sucrose density gradient column. After centrifugation for 90 minutes at 12590 rpm in a Spinco E ultracentrifuge with SW-39 rotor the column was divided in eight fractions of equal volume from top to the bottom and the hemagglutinating and hemolytic activities of each fraction were determined.

As shown in Fig. 8 the major peak of the hemagglutinating activity is localized in the same position as that of hemolytic activity and the distribution of both activities is in fairly good agreement. However, while the upper boundaries of both peaks coincide the lower boundaries show marked discrepancy. The reason for this is not clear but there is a possibility that the hemolytic activity is affected by the high concentration of sucrose in the medium. The result for influenza virus, PR8 strain, is also shown in Fig. 8 (dotted line) as a control. The virus preparation used seems to be almost homogeneous even from another mean of analysis in a centrifugal field.

DISCUSSION

On heat treatment, the infectivity of HVJ is first lost, and then the hemolytic activity. Hemagglutinating activity is most resistant to heat inactivation. With

Fig. 8. Distribution of hemagglutinating and hemolytic activities on HVJ in a sucrose density gradient column.



△··· hemagglutinating activity ⊙— hemolytic activity
Dotted line shows the distribution of hemagglutinating activity of influenza virus, PR 8 strain, used as a control.

ultra-violet irradiation, inactivation occurs in the same order. On the other hand it is known that ionizing radiation, such as the gamma rays emitted from Co^{60}, inactivate the viral elements in proportion to their sensitive volumes.

Gamma ray inactivation experiments on HVJ showed that the infectivity of the virus is inactivated first and then, at the same rate the hemagglutinating and hemolytic activities. The diameters of the sensitive parts for infectivity, hemagglutinating activity, and hemolytic activity assuming them to be sphere were 38, 10 and 10 millimicrons respectively, as calculated from 37 per cent doses of irradiation.

The coincidence of the inactivation curves for hemagglutinating and hemolytic activities, however, might be only apparent. The results of Morgan *et al.* and Hosaka showed that the hemolysis is caused by an enzymatic reaction of the viral elements and the binding between the enzyme and its substrate is essential for the development of enzymatic activity, hemagglutination may preceed the hemolysis during hemolysis.

From preliminary experiments, it appears that hemolysis is proportional to the concentration of virus, hemagglutinating titer. Therefore a value of 10 millimicron as the diameter of the sensitive part for hemolytic activity might be only

an apparent value.

As isolated hemagglutinin was inactivated in a similar way to the native virus an estimate of the sensitive part of hemagglutinating activity is acceptable. But, on the other hand, as all attempts to obtain subunits which bears hemolytic activity from virus particles were unsuccessful, an estimate of the hemolytic agent was not obtained. It has been reported that the hemolytic activity resides on the virus particle itself, and the density gradient fractionation experiment above described confirmed this.

In electronmicrographs HVJ apparently pleomorphic but the survival curve suggests that the infective center of the virus is highly homogeneous and a little larger than that of the influenza virus. This is consistent with sedimentation constant measurements and from the sucrose density gradient fractionation experiment.

It is interesting that the isolated hemagglutinin does not have any so-called secondary effect of radiation in suspension while the hemagglutinating and hemolytic activities of native virus do show secondary effect in suspension.

The hemolytic activity of native HVJ is greatly increased when the virus is freeze-dried and then redissolved. The cause of this enhancement in hemolytic activity may be the same as those already reported on freezing and thawing or osmotic effects on this virus.

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